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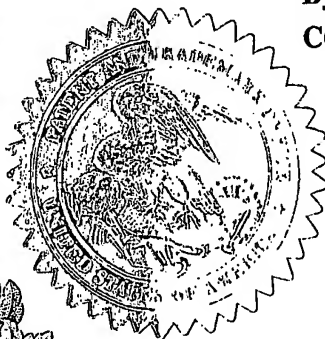
**APPLICATION NUMBER: 60/407,843**

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PROVISIONAL APPLICATION FOR PATENT  
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Case No VANM260.001PRF  
Date: August 30, 2002  
Page 1

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ATTENTION: PROVISIONAL PATENT APPLICATION

Sir:

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR § 1.53(c).

For: **MYROTHECIUM SP. TRANSFORMATION AND EXPRESSION SYSTEM**

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Enclosed are:

- (X) Specification in 47 pages.
- (X) 4 sheets of drawings.
- (X) A check in the amount of \$160 to cover the filing fee is enclosed.
- (X) A return prepaid postcard.
- (X) The Commissioner is hereby authorized to charge any additional fees which may be required, now or in the future, or credit any overpayment to Account No. 11-1410.

Was this invention made by an agency of the United States Government or under a contract with an agency of the United States Government?

- (X) No.
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PROVISIONAL APPLICATION FOR PATENT  
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Case No. VANM260.001PRF

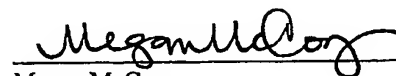
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Page 2

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**CERTIFICATE OF MAILING BY "EXPRESS MAIL"**

Attorney Docket No. : VANM260.001PRF  
Applicant(s) : Jonniaux, et al.  
For : MYROTHECIUM SP. TRANSFORMATION  
AND EXPRESSION SYSTEM  
Agent : Megan McCoy  
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**MYROTHECIUM SP. TRANSFORMATION AND EXPRESSION SYSTEM****Field of the invention**

[0001] The present invention is related to a method to express proteins or to modify protein expression in a microbial host.

**Background of the invention**

[0002] Since the advent of the genetic engineering a number of living cells has been transformed with DNA in order to produce homologous or heterologous proteins or to modify their metabolism by introducing for example new metabolic pathways. The variety of cloning hosts spans from bacteria such as *Escherichia coli* till human cells and comprises organisms as diverse as yeasts, fungi or insect cells.

[0003] Among these organisms, the filamentous fungi have been the subjects of many studies. Indeed some of these organisms are industrially important, such as *Aspergillus* sp., *Trichoderma* sp. or *Fusarium* sp.

[0004] The first report of a DNA-mediated transformation of a filamentous fungi was published in 1973 using *Neurospora crassa* (Mishra, N.C. and Tatum E.L., 1973. Non-Mendelian inheritance of DNA-induced inositol independence in *Neurospora*. Proc. Nat. Acad. Sci. USA. 70:3875-3879). Later on, a protoplast transformation system has been developed for the same organism (Case et al, 1979. Efficient transformation of *Neurospora crassa* by utilising hybrid plasmid DNA. Proc. Nat. Acad. Sci. USA. 76:5259-5263). Transformation of *Podospira anserina* (Stahl et al, 1982. Replication and expression of a bacterial-mitochondrial hybrid plasmid in the fungus *Podospira anserina*.

Proc. Nat. Acad. Sci. USA. 79:3641-3645) and *Aspergillus nidulans* (Tilburn et al, 1983. Transformation by integration in *Aspergillus nidulans*. Gene. 26:205-221; Ballance et al, 1983. Transformation of *Aspergillus nidulans* by the orotidine-5'-phosphate decarboxylase gene of *Neurospora crassa*. Biochem. Biophys. Res. Comm. 112:284-289) were also reported. The same techniques have been successfully used to transform the commercially important industrial fungi *Aspergillus niger* (Buxton et al, 1985. Transformation of *Aspergillus niger* using the *argB* gene of *Aspergillus nidulans*. Gene. 37:207-214), *Aspergillus oryzae* (Gomi et al, 1987. Agric. Biol. Chem. 51:2549-2555) and *Trichoderma reesei* (Penttillä et al, 1988. Gene. 61:155-164).

[0005] Today transformations systems have been reported for many fungal classes (see for example the chapter on Genetic manipulation of fungi by transformation by Lemke P.A. and Peng M. in The Mycota volume II Genetics and Biotechnology, Kück ed, Springer-Verlag, 1995).

[0006] Some of these are patented (see for example patent EP0184438B1 on "Transforming *Aspergillus* and plasmids for use therein" or patent application WO9602653 on "Thermophilic fungal expression system"). Also are described parts of transformation systems such as the use of promoters or plasmids (For example EP0489718 "Process for the production of protein products in *Aspergillus oryzae* and a promoter for use in *Aspergillus*").

[0007] Enzymes are proteins that are produced by all living organisms. They are highly specific biological catalysts that speed up chemical reactions selectively. [0008] They are produced industrially for a number of application ranging from detergent formulation to food application (baking, fruit processing,...) (for reviews see the books of Uhlig, H., Industrial enzymes and their applications, 1998, John Wiley and

sons, Inc; Enzymes in food technology, 2002, Whitehurst, R.J. and Law, B.A. eds, Sheffield Academic Press; Industrial enzymology, 1996, Godfrey, T. and West, S. eds., Macmillan Press ltd).

[0009] About 90% of all enzymes used in industrial processing are produced by fermentation of microorganisms.

[0010] Today very few species of microorganisms are used for industrial enzyme production. This is a limitation imposed by producing companies needing the widest market range for their products, including food processing.

[0011] However, this situation is changing as new opportunities outside food processing are appearing. Also the increasing introduction of productions using genetically manipulated microorganisms strengthens the need to find new organisms to be used. The genetic engineering allows also the possibility to produce enzymes naturally produced by microorganisms having poor growing properties in fermentors.

[0012] Besides enzyme production, genetically modified microorganisms are used for many other applications such as but not restricted to therapeutical drugs production (antibiotics, anticancer,...), new metabolic pathway expression,...

[0013] Therefore, there is still a need for expression systems that would allow high levels of production of various compounds.

[0014] The diversity of the microbial world is today largely underexploited. It has been estimated that less than 1% of the microorganisms living on earth have already been discovered. Among those identified, very few have been the subject of investigations other than morphological or taxonomical ones.

[0015] It is therefore the subject of the present invention to present the results of a screening of this microbial diversity and to characterise a microbial genus that

would present the characteristics required for a performing cloning host cell: good growth in industrial conditions, transformability, high expression level or well modulated expression level of homologous or heterologous genes or gene sets.

### Short description of the drawings

[0016] Figure 1 shows a phylogenetic tree constructed with fungal ITS sequences available in the NCBI database or provided in the present invention.

[0017] Figure 2 shows a partial nucleotide sequence and its deduced protein sequence of the *Myrothecium gramineum* MUCL39210 glyceraldehyde-3-P dehydrogenase gene

[0018] Figure 3 shows a multiple sequence alignment of four partial GPD protein sequence from the strains MUCL39210, MUCL11831, CBS449.71 and IMI290405.

[0019] Figure 4 shows an agarose gel electrophoresis of the DNA fragments amplified by PCR from *Myrothecium* transformants and corresponding to a part of the *Aspergillus oryzae*  $\alpha$ -amylase reporter gene (odd lanes from 5 to 13) and to a part of the hygromycin selection gene (even lanes from 4 to 12). Lanes 2-3 and 14-15 correspond respectively to negative and positive controls.

### Detailed description of the invention

[0020] A first aspect of the invention is the screening of a collection of fungi. The collection screened in the present invention is the collection from the MUCL ("Mycothèque de l'Université Catholique de Louvain", Place Croix du Sud, 2, B-1348 Louvain-la-Neuve, Belgium). However, it will be obvious for a person skilled in the art that other public or private



microorganisms collections can be screened following the same approach to provide the selected genus with the required properties. Examples of public collections where fungal strains are available are ATCC (American Type Culture Collection - P.O. Box 1549, Manassas, VA 20108, USA), CBS (Centraalbureau voor Schimmelcultures - P.O.Box 85167, 3508 AD Utrecht, The Netherlands), DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH - Mascheroder Weg 1 b, D - 38124 Braunschweig, Germany), IMI (International Mycological Institute - Bakeham Lane, Egham, Surrey TW20 9TY, UK),....

[0021] 3.000 different species or strains of fungi were first selected from the MUCL collection based on several criteria such as but not restricted to their non-pathogenicity or the fact that no transformation for this species has been reported in the scientific literature or in a patent. All these strains were grown in liquid culture and on solid medium. Useful liquid media are rich media containing for example glucose, peptone, yeast extract, malt extract, soy meal,.... Useful solid media are for example PDA (Potato Dextrose Agar), MA (Malt Extract Agar). The first selection criteria were based on the growth rate, the biomass production, the protein production, the enzyme production (xylanase, amylase and proteases) and the morphology of the fungal strains.

[0022] The growth rate can be usefully determined for example by measuring the rate of increase of the growth diameter of a single colony on a solid medium.

The biomass production can be usefully determined by evaluating for example the dry matter of cells collected by filtration or by centrifugation at various time intervals during the culture.

[0023] The protein production can be usefully determined, using methods well known by persons skilled in the art, by measuring the total amount of proteins in a culture supernatant.

Examples of such methods are the Lowry (Lowry O.H. et al. 1951. J. Biol. Chem. 193:265) or the Bradford (Bradford M.M. 1976. Anal. Biochem. 72:248) methods.

[0024] The enzyme production can be usefully determined by measuring the amount of enzyme produced in a culture supernatant using methods well known by persons skilled in the art. Examples of methods of xylanase activity determination are the measure of the release of reducing sugar from xylan (Miller G.L. 1959. Anal. Chem. 31:426) or the measure of the release of coloured compounds from modified substrates (for examples AzowAX or Xylazyme AX from Megazyme). Amylase activity can be determined for example by measuring the rate of release of reducing sugar from starch (Miller, op.cit.) or by using specific commercially available kits (amylase kit from Sigma; Amyl kit from Roche Diagnostics,...). Protease activity can be determined for example using commercially available kits (Azocasein from Megazyme,...).

[0025] The morphology of the fungal strains can be advantageously studied by examining portions of the cultures with a microscope (light microscope, electronic microscope,...).

[0026] From this screening were retained about one tenth of the 3.000 fungal species or strains. Among the selected strains, about 80 strains were chosen based on their good protein production, about 80 based on their good biomass production, about 60 based on their good amylase production, about 60 based on their good xylanase production and about 10 based on their good protease production.

[0027] The selected strains were submitted to a second round of screening in liquid cultures using five different culture media, each one having a particular purpose. The first medium has been selected for maximal protein production, the second for maximal biomass production, the third for the amylase induction,

the fourth for xylanase induction and the last one for the protease induction.

[0028] Typical media for protein and/or biomass production are rich media composed for example of carbon and nitrogen sources such as glucose, malt extract, peptone, soy meal,... A typical medium for amylase production contains usually starch or starch derivatives as enzyme inducer. A typical medium for xylanase production usually contains an hemicellulose-rich inducer such as but not restricted to xylan, wheat straw or wheat bran. A typical medium for protease production is usually enriched in various proteins or amino acids sources.

[0029] The screening criteria retained were the same as for the first step: the growth rate, the biomass production, the protein production, the production of amylase, xylanase and/or protease and the morphology.

[0030] A statistical approach was followed to identify the strains that ranked with a high score for one or several criteria in more than one culture medium. About 50 strains were selected out from this second screening.

[0031] In order to eliminate those strains that would produce any antimicrobial compounds, the production of such compounds by the selected strains has been checked.

The inhibition of growth of some bacterial species has been tested using the method described by Färber et al (Färber P. and Geisen R. 1994. Antagonistic activity of the food-related filamentous fungus *Penicillium nalgiovense* by the production of penicillin, Appl. Environ. Microbiol. 60:3401-3404). Indicator strains were the following: *Micrococcus flavus* (MUCL collection, determined with API 20E), *Bacillus subtilis* DSM6633, *Bacillus cereus* LMG6923, *Staphylococcus aureus*, *Salmonella* sp. (MUCL collection, determined with API 20E), *Escherichia coli*, *Proteus mirabilis* (MUCL collection, determined with API 20E),

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*Candida blankii* MUCL29808. These strains have been chosen according to Färber et al (op. cit.), to Aissaoui & al (Aissaoui H., Agut M., Torras M. 1999. Effect of the raw extract of *Arthrinium* strains (Hyphomycetes, Dematiaceae) on the growth of pathogenic bacteria in poultry feed. *Microbios.* 100(396):109-115) and to the Oxoid Manual (7<sup>th</sup> edition 1995). It is also obvious for a person skilled in the art that other bacterial species or strains can equally be used for the evaluation.

[0032] Potential producers of antibacterial compounds have been identified and discarded from the list of selected strains.

[0033] A similar method has been used to control the production of antifungal compounds using *Botrytis cinerea* MUCL30834, *Fusarium moniliforme* MULC14280 and *Alternaria alternata* MUCL16089 (Mangiarotti A., Frate G., Picco AM., Caretta G. 1987. Antagonistic activity in vitro of some saprophytic fungi occurring on the phylloplane of rice, wheat and maize. *Boletin Micologico.* 3:183-189). Again the potential antifungal compounds producers were discarded from the list.

[0034] 30 of the remaining strains (the best ranking in the statistical analysis) were then tested for their ability to incorporate foreign DNA as well as to express a reporter gene. The general method of protoplasts transformation (Punt P.J. and van den Hondel C.A.M.J.J. 1992. Transformation of filamentous fungi based on hygromycin B and phleomycin resistance markers, *Methods in Enzymology.* 216:447-457) has been followed. For each strain several parameters, that a person skilled in the art will recognise as critical, were investigated. Among these parameters were:

- the possibility to use one or several of the common transformation markers (hygromycine B resistance gene, phleomycin resistance gene, phosphinothricine resistance gene, acetamidase gene, pyrG gene, argB gene, niaD gene,...)

- the conditions to obtain viable protoplasts in large amounts (culture medium, culture temperature, length of the culture, type of digesting enzymes, duration of cell walls digestion, type of osmotic stabiliser,...
- the conditions of transformation.
- the conditions of protoplasts regeneration.

[0035] A useful reporter gene is the well-known gene coding for the TAKA-amylase of *Aspergillus oryzae*. The flanking regions of the coding region (essentially the promoter and the terminator sequences) or flanking regions of the coding region from others genes are advantageously associated with the coding region to provide a complete expression cassette. The sequence of the TAKA-amylase gene is publicly available (Tada, S., Iimura, Y., Gomi, K., Takahashi, K., Hara, S. and Yoshizawa, K. 1989. Cloning and nucleotide sequence of the genomic Taka-amylase A gene of *Aspergillus oryzae*. Agric. Biol. Chem. 53:593-599) and the corresponding DNA can be easily cloned using for example PCR techniques.

[0036] It is therefore the object of the present invention to show that the genus *Myrothecium* presents all the characteristics needed for a performing cloning host such as but not limited to good transformability, good expression of homologous and heterologous genes or genes sets, easy metabolic pathway engineering, good performances in fermentation, poor proteases production, ...

[0037] In a specific aspect of this embodiment, it is more precisely shown that the species *Myrothecium gramineum* (syn. *Xepiculopsis graminea*) presents all the characteristics needed for a performing cloning host such as but not limited to good transformability, good expression of homologous and heterologous genes or genes sets, easy metabolic pathway engineering, good performances in fermentation, poor proteases production, ...

[0038] In a more specific aspect of this embodiment, it is more precisely shown that the strain *Myrothecium gramineum* (syn. *Xepiculopsis graminea*) MUCL39210 presents all the characteristics needed for a performing cloning host such as but not limited to good transformability, good expression of homologous and heterologous genes or genes sets, easy metabolic pathway engineering, good performances in fermentation, poor proteases production, ...

[0039] The genus *Myrothecium* is defined in the following way:

"Fructification cupulate, sporodochial or synnematosus, formed from closely compacted conidiophores arising from a more or less developed stroma and bearing a mass of slimy green to black spores which becomes hard on drying. Fructification surrounded by differentiated marginal hyphae which may be free or laterally compacted into a plectenchymatous wall. Hyaline or darkened setae sometimes present arising from the basal stroma. Conidiophores hyaline, olivaceous or slightly darkened, macronematous, irregularly and repeatedly branched forming several branches at each node, the ultimate branches bearing the conidiogenous cells (phialides) in whorls. Phialides hyaline or darkened at the apex, occasionally percurrent sometimes with a flared collarete, compacted into a dense parallel layer. Spores unicellular, hyaline or dilute olivaceous, black in mass, slimy" (Tulloch M., 1972, The genus *Myrothecium* Tode ex Fr. Mycological Papers n°130, Commonwealth Mycological Institute, Kew, Surrey, England).

[0040] The genus *Myrothecium* includes i.a. the species *Myrothecium inundatum*, *Myrothecium prestonii*, *Myrothecium leucotrichum*, *Myrothecium cinctum*, *Myrothecium masonii*, *Myrothecium roridum*, *Myrothecium verrucaria*, *Myrothecium*

*carmichaelii*, *Myrothecium lachastrae*, *Myrothecium atrum*,  
*Myrothecium atroviride*, *Myrothecium gramineum*,...

[0041] The species *Myrothecium gramineum* or its synonymous  
species *Xepiculopsis graminea* has the following description:

"Foliicolous. Conidiomata stromatic, scattered to gregarious,  
seemingly superficial, discoid to cupulate, excipulate with a  
dense white fringe of long white setae and sterile elements with  
coiled apices surrounding a central greenish-black agglutinated  
conidial mass, 220-450  $\mu\text{m}$  diam., 150-190  $\mu\text{m}$  deep (excluding  
setae); basal stroma moderately developed, composed of a  
colourless textura angularis in the lower layers gradually  
merging with textura prismatica above; excipulum well-developed,  
of compact and colourless textura prismatica to textura  
porrecta; marginal hyphae with terminal cells 7-16 x 2-3  $\mu\text{m}$ , of  
two kinds: those on the inside subcylindrical with blunt apices,  
olivaceous to fuliginous, irregularly tuberculate, and those on  
the outside with a subcylindrical, olivaceous to fuliginous,  
irregularly tuberculate basal part, and a slender, colourless,  
smooth, sinuate apical process up to 47  $\mu\text{m}$  long and 1  $\mu\text{m}$  wide.

[0042] Conidiomatal setae sparse arising from outer cells  
of the excipulum, unbranched, straight or slightly incurved,  
subulate, thick-walled with continuous narrow lumen, smooth-  
walled, colourless, up to 450  $\mu\text{m}$  long, 15-20  $\mu\text{m}$  wide at the  
broadest point, up to 1.5  $\mu\text{m}$  wide at the acute apex.  
Conidiophores arising from the uppermost cells of the basal  
stroma and inner layer of cells of the excipulum in the  
concavity of the conidiomata, unbranched or branched, septate,  
colourless to almost colourless, smooth. Conidiogenous cells  
discrete, occasionally integrated, subcylindrical to clavate  
with flared collarettes and moderate to marked apical periclinal  
thickenings, more or less pale olivaceous in the upper part with  
a deeper pigmentation near the collarettes, smooth, (7-)12-16 x

2-3 [mean =  $14 \times 2.5$ ]  $\mu\text{m}$ . Conidia fusiform to ellipsoid with an acute apex and a narrow truncate base, unicellular, pale olivaceous, smooth,  $6.5-12.5 \times 2-3(-3.5)$  [mean =  $9 \times 2.5$ ]  $\mu\text{m}$ , with an apical mucoid appendage; mean conidium length/width ratio = 3.6:1. (NAG RAJ T.R. 1993. Coelomycetous anamorphs with appendage-bearing conidia, published by Mycologue Publication)

[0043] It should be understood that in the present invention the terms *Myrothecium sp.*, *Myrothecium gramineum* or *Xepiculopsis graminea* refer not only to those organisms present currently under these names in the various public or private microorganisms collections around the world but also to the species which have previously been or currently are assigned to other species but that possess the same morphological and cultural characteristics as defined above.

[0044] The scope of the present invention also includes mutant strains of *Myrothecium sp.*, *Myrothecium gramineum* or *Xepiculopsis graminea*. Types of mutants include but are not restricted to auxotrophic mutants, protease(s) deficient mutants, secretion mutants, constitutive mutants, deregulated mutants. Mutants can be obtained using techniques well known by persons skilled in the art.

[0045] In another embodiment of this invention, it will be referred to rDNA sequences or ITS sequences to assign a particular strain to a species or a genus.

[0046] The internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA) has been widely used to reconstruct microbial phylogeny. The ITS region separating 18s and 26s nrDNA and the 5.8s coding region sequence has been widely used to characterise interspecific and intergenic levels divergences. The ITS region is therefore useful to gain insight into DNA sequence evolution. This is due to the fact that, as a transcribed, but untranslated sequence, it is free to vary.



[0047] Using a combination of the morphological taxonomical methods and the ITS sequences determination and comparison, the inventors have shown that some of the strains deposited as *Myrothecium gramineum* (or as its synonymous *Xepiculopsis graminea*) in public culture collections have been erroneously identified as *Myrothecium gramineum* or *Xepiculopsis graminea*. They have also shown that representative members of other species of the genus *Myrothecium* also present the characteristics needed for a performing cloning host such as but not limited to good transformability, good expression of homologous and heterologous genes or genes sets, easy metabolic pathway engineering, good performances in fermentation, poor proteases production, ...

[0048] Other species of the genus *Myrothecium* are therefore considered as integral part of the present invention.

[0049] *Aspergillus* species and in particular *Aspergillus niger* have been used since years as cell factories to produce homologous and heterologous proteins and can therefore be considered as the models for gene expression in filamentous fungi (see for example the review van Gorcom R.F.M., Punt P.J., van den Hondel C.A.M.J.J. 1994. Heterologous gene expression in *Aspergillus* in The genus *Aspergillus*: from taxonomy and genetics to industrial applications Powell K.A., Renwick, A., Peberdy J.F. eds. Plenum Press, New York, pp 241-250).

[0050] It is therefore one of the objects of the present invention to show that strains from the selected filamentous fungi genus produce, when transformed with homologous or heterologous DNA more protein and/or enzyme than *Aspergillus niger* in the same conditions.

[0051] In another embodiment of the present invention, it is provide DNA constructs that, when used to transform *Myrothecium sp.*, *Myrothecium gramineum* or *Xepiculopsis graminea*,

allow the expression of the homologous or the heterologous gene present on this DNA construct. A particular result of the present invention is that the DNA when introduced in the host cell, integrates in the host cell chromosome. This phenomenon is similar to what has already been described for many other species of fungi. Therefore, it is obvious for a person skilled in the art that the same type of constructs as the one used for other species of filamentous fungi can be used for *Myrothecium sp.*, *Myrothecium gramineum* or *Xepiculopsis graminea*. For example, the DNA constructs that are useful in the transformation of *Aspergillus* species such as *Aspergillus niger*, *Aspergillus nidulans* or *Aspergillus oryzae* are also useful for the purpose of the present invention. The promoter may be any DNA sequence that induces a transcriptional activity in the host and may be derived from any homologous or heterologous gene. Examples of promoters are the *Aspergillus oryzae* TAKA-amylase promoter, the promoters of glycolytic enzymes genes such as GPD, PGK, ADH. These promoters may be regulatable or not. Transcription terminator and polyadenylation sequences may also be added to the DNA constructs.

[0052] In a particular aspect of this embodiment, it is also provided additional tools to enhance the gene expression in the selected host strains, namely *Myrothecium sp.*, *Myrothecium gramineum*, or *Xepiculopsis graminea*. Using methods well known by persons skilled in the art, the gene coding for the glyceraldehyde 3-phosphate dehydrogenase (GPD gene) has been cloned as well as its flanking regions (promoter and terminator). These regions are usefully used to make DNA constructs that give a better expression in the selected host strains.

[0053] In a further aspect of this embodiment, the DNA constructs may comprise particular sequences, such as sequences

coding for a leader or a signal peptide, that allows the protein of interest to be secreted in the extracellular medium. This preregion may be derived from any secreted protein, or may be synthesised using consensus sequences, provided that this preregion allows the secretion of the desired protein.

[0054] In yet another aspect of this embodiment, the DNA constructs may comprise genes to allow the selection of the transformants of *Myrothecium* sp., *Myrothecium gramineum* or *Xepiculopsis graminea*. Those selection markers are for example but are not restricted to the acetamidase gene (amdS) of *Aspergillus* sp. or the hygromycine resistance gene (hygB) of *Escherichia coli*. Other markers include the argB, niaD, trpC or pyrG genes of *Aspergillus* sp.

[0055] In yet another aspect of this embodiment, the various elements of the DNA constructs may be either incorporated in the same vector or plasmid or DNA fragment or may be included in separate vectors, plasmids or DNA fragments provided that the DNA sequences required for gene expression are associated with their respective coding sequence.

[0056] An example of suitable selection vector is the plasmid pCSN43 that contains the hph gene of *Escherichia coli* under the control of *Aspergillus* transcriptional signals (Staben C. et al. 1989. Use of a bacterial hygromycine B resistance gene as a dominant selectable marker in *Neurospora crassa* transformation. Fungal Genetics Newsl. 36:79). It confers to transformed cells resistance to hygromycine B.

[0057] Another example of suitable selection vector is the plasmid p3SR2 that contains the acetamidase gene from *Aspergillus nidulans* (Hynes et al. 1983. Isolation of genomic clones containing the amdS gene of *Aspergillus nidulans* and their use in the analysis of structural and regulatory mutations. Mol. Cell. Biol. 3:1430). It confers to transformed

cells the ability to grow on a minimal medium containing acetamide as sole nitrogen source.

[0058] In another embodiment of the present invention, it is provide a method to transform *Myrothecium* sp., *Myrothecium gramineum* or *Xepiculopsis graminea*. The preferred method of transformation is the standard protoplast transformation. In this method, the DNA constructs are incubated in particular conditions with protoplasts of the host strain. The transformants are first selected on the basis of the selection marker used (for example amdS or hygB) and in a second step cotransformants that have integrated the desired gene are screened.

It is one of the objects of the present invention to show that, when transformed with two separate DNA constructs (containing for example a selection marker and a reporter gene), most of the transformants of *Myrothecium*, *Myrothecium gramineum* or *Xepiculopsis graminea* have integrated simultaneously both DNA constructs.

[0059] Other methods of transformation can usefully replace the protoplasts transformation and are well known by persons skilled in the art. Examples of alternative methods are the electroporation, the transformation by particle bombardment or the transformation by *Agrobacterium* sp.-mediated recombination.

[0060] In another embodiment of the present invention, it is provided *Myrothecium* sp., *Myrothecium gramineum* or *Xepiculopsis graminea* transformed strains that express or overexpress a particular homologous or heterologous gene or a particular set of genes.

[0061] In another embodiment of the present invention, it is provided *Myrothecium* sp. *Myrothecium gramineum* or

Xepiculopsis graminea genetically engineered strains with one or several altered metabolic pathways.

[0062] In another embodiment of the present invention it is provided culture media to cultivate the *Myrothecium sp.*, *Myrothecium gramineum* or *Xepiculopsis graminea* transformed strains. A typical culture media contain carbon (C) sources and/or nitrogen (N) sources and/or salts and/or secondary metabolites and/or miscellaneous compounds. C sources could be glucose, saccharose, ... as well as complex substrates like arabinoxylan, pectin, ... as well as lipids and organic acids. N sources could be amino acids, nitrate, ammonia, ... as well as complex substrates like yeast extract, potato extract, soy extract, ...

[0063] Salts could be chosen among phosphate salts, Magnesium salts, potassium salts, .... Secondary metabolites could be antibiotics, mycotoxins, hormones, vitamins,.... Miscellaneous compound could be defoamers, antifoamers, emulsifiers, ...

[0064] In another embodiment of the present invention it is provided culture techniques to cultivate the *Myrothecium sp.*, *Myrothecium gramineum* or *Xepiculopsis graminea* transformed strains. Examples of such culture techniques are liquid cultures in erlenmeyer flasks or fermentor, solid state fermentation, airlift fermentation,...

[0065] This invention will be described in further details in the following examples by reference to the enclosed drawings, which are not in any way intended to limit the scope of the invention as claimed.

# EXAMPLE 1

## Characterisation of the *Myrothecium* strains

### 1.1. Molecular characterisation of the *Myrothecium* strains

[0066] The genomic DNA of the strains currently assigned as *Myrothecium gramineum* (or of the synonymous strain *Xepiculopsis graminea*) MUCL39210, MUCL11831, CBS449.71, IMI140595 and IMI290405 has been prepared using the Dneasy Plant Mini Kit (Qiagen).

[0067] The DNA coding for RNA ITS (Internal Transcribed Spacer) and 28S regions were analysed by PCR. The oligonucleotides used for the 28S region were: LR0R (5'-ACCCGCTGAACTTAAGC-3'), LR6 (5'-CGCCAGTTCTGCTTACC-3'), LR3R (5'-GTCTTGAAACACGGACC-3') and LR3 (5'-CCGTGTTTCAAGACGGG-3'). The oligonucleotides used for the ITS region were ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3').

[0068] PCR reactions were performed in 0.25 ml tubes, using a MJ Research PTC-200 Peltier Thermal Cycler. PCR reactions (50 µl) contained approximately 20 ng of fungal genomic DNA (in 10 µl), 10 pM ITS1 and ITS4 primer pairs (1 ml), 100 mM dNTPs (1 µl), 1X reaction buffer (10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl.sub.2, 50 mM KCl, 0.1 ng/ml BSA) (5 µl), and 2.5 U Taq polymerase (Invitrogen) (0.5 µl). Thermal cycling conditions were as follows: for 28S: initial denaturation (94°C, 5 min), followed by 35 cycles of denaturation (94°C, 1 min), annealing (50°C, 1 min 30 s), primer extension (72°C, 4 min), and one final cycle of primer extension (72°C, 10 min); for ITS: initial denaturation (94°C, 3 min), followed by 30 cycles of denaturation (94°C, 1 min 30 s), annealing (55°C, 1 min 30 s), primer extension (72°C, 2 min), and one final cycle of primer extension (72°C, 10 min).

[0069] The reaction products (10  $\mu$ l) were analysed by electrophoresis on a 0.8 % agarose gel in Tris-acetate EDTA buffer (TAE buffer) to check the presence of a unique amplified DNA fragment. The PCR product was then purified using PCR purification kit (Qiagen) and quantified with a Biophotometer (Eppendorf).

[0070] DNA sequencing reactions were performed in 0.25 ml tubes, using a MJ Research PTC-200 Peltier Thermal Cycler. PCR reactions (20  $\mu$ l) contained 25 to 100 fmol of purified PCR product (0.5 to 10  $\mu$ l), 1.6  $\mu$ M of -47 sequencing primer (2  $\mu$ l), 8  $\mu$ l of DTCS Quick Start Master Mix (100  $\mu$ l of dNTPs, 200  $\mu$ l of ddATP, 200  $\mu$ l of ddGTP, 200  $\mu$ l of ddCTP, 200  $\mu$ l of ddUTP, 200  $\mu$ l of 10X reaction buffer and 100  $\mu$ l Taq polymerase). Thermal cycling conditions were as follows: 30 cycles of denaturation (96°C, 20 sec), annealing (50°C, 20 sec), primer extension (60°C, 4 min), followed by holding at 4°C. The reaction is stopped by adding 4  $\mu$ l of Stop Solution (1.5M NaOAc + 50 mM EDTA prepared fresh daily by mixing equal volumes of 3M NaOAc and 100mM EDTA) and 1  $\mu$ l of 20 mg/ml glycogen. The sequencing reactions were transferred to a 96 wells plate. 60  $\mu$ l of 95% cold ethanol from -20°C freezer were added, the plate was sealed with adhesive foil, mixed thoroughly, incubated at -20°C during 10 minutes, centrifuged at 14.000 rpm at 4°C during 30 minutes and the supernatant was carefully removed. The pellets were then rinsed two times with 200  $\mu$ l of 70% cold ethanol. Finally, the samples in the plate were dried under vacuum until no traces of ethanol remained (5 to 15 minutes). The samples were resuspended in 40  $\mu$ l of Sample Loading Solution, left at room temperature for ten minutes then mixed thoroughly and overlaid with one drop of light mineral oil (Sigma Cat# M 5904).

[0071] The sequencing was done in a capillary electrophoresis sequencer "CEQ™ 2000 XL DNA Analysis System"

(Beckman Coulter). The sequences generated were rectified and aligned with the software Sequencher™ 4.1.

The sequence of 28s rDNA of the strain MUCL39210 is shown in SEQ ID NO 1.

[0072] The sequence of 28s rDNA of the strain MUCL11831 is shown in SEQ ID NO 2.

[0073] The sequence of 28s rDNA of the strain CBS449.71 is shown in SEQ ID NO 3.

[0074] The sequence of 28s rDNA of the strain IMI140595 is shown in SEQ ID NO 4.

[0075] The sequence of 28s rDNA of the strain IMI290405 is shown in SEQ ID NO 5.

[0076] The ITS sequence of the strain MUCL39210 is shown in SEQ ID NO 6.

[0077] The ITS sequence of the strain MUCL11831 is shown in SEQ ID NO 7.

[0078] The ITS sequence of the strain CBS449.71 is shown in SEQ ID NO 8.

[0079] The ITS sequence of the strain IMI140595 is shown in SEQ ID NO 9.

[0080] The ITS sequence of the strain IMI290405 is shown in SEQ ID NO 10.

[0081] These sequences were compared and compared with other closely related sequences of the NCBI gene bank (<http://www.ncbi.nlm.nih.gov/BLAST/>). The sequences were first aligned using the software ClustalX 1.5b and then a bootstrap tree was constructed using the software PAUP 4.0b10.

[0082] A phylogenetic tree with relevant data is presented on figure 1. It is concluded from this tree that some of the investigated *Myrothecium gramineum* (syn. *Xepiculopsis graminea*) strains have been erroneously assigned to a particular species.



However, all the strains studied, with the exception of MUCL11831 belong to the *Myrothecium* genus.

### 1.2. Morphological identification of the *Myrothecium* strains.

[0083] A taxonomical reidentification of the MUCL39210 strain has confirmed that it presents all the morphological characteristics of the species *Myrothecium gramineum* (syn. *Xepiculopsis graminea*) (see the description in the detailed description of the invention).

[0084] A similar examination of the strain MUCL11831 confirmed that this strain does not belong to the *Myrothecium* genus.

## EXAMPLE 2

### Transformation of *Myrothecium*

#### 2.1. Choice of selectable markers

[0085] *Myrothecium gramineum* (syn. *Xepiculopsis graminea*) MUCL39210 was incubated on PDA (Potato Dextrose Agar), AMMA (Aspergillus Minimal Medium Agar) or MA2 (Malt Extract Agar) plates with increasing concentration of hygromycin B, phleomycin or phosphinothricine. The threshold antibiotic concentration where no growth occurred was 500 µg/ml for hygromycin B, >100 µg/ml for phleomycin and > 400µg/ml for phosphinothricin.

[0086] *Myrothecium gramineum* (syn. *Xepiculopsis graminea*) MUCL39210 was incubated on minimal medium plates containing acetamide as sole nitrogen source. The strain did not grow on this medium.

[0087] Similar results were obtained with the strains of *Myrothecium gramineum* CBS449.71, IMI140595, IMI290405 and *Myrothecium verrucaria* CBS328.52

## 2.2. Vectors

### 2.2.a. Selectable vector

[0088] The vector chosen to allow the selection of the transformants by their resistance to an antibiotic is the plasmid pCSN43 that contains the hph gene of *Escherichia coli* under the control of *Aspergillus* transcriptional signals (Staben C. et al. 1989. Use of a bacterial hygromycin B resistance gene as a dominant selectable marker in *Neurospora crassa* transformation. Fungal Genetics News1. 36:79).

[0089] The vector chosen to allow the selection of the transformed cells by their ability to grow on a minimal medium containing acetamide as sole nitrogen source is the plasmid p3SR2 (Hynes et al. 1983. Isolation of genomic clones containing the amdS gene of *Aspergillus nidulans* and their use in the analysis of structural and regulatory mutations. Mol. Cell. Biol., 3:1430)

### 2.2.b. Expression vectors

#### 2.2.b.1. p2G-S

[0090] A DNA fragment covering the coding region as well as its terminator region was amplified by a PCR reaction. The synthetic oligonucleotide AMY1 (5'- GGAATTCCACAGAAGGCATTTATG - 3') was chosen to contain the ATG codon of the first amylase methionine. The synthetic oligonucleotide AMY2 (5'-

GCTCTAGAGCAACCACCAGGTCA - 3') corresponds to the sequence located about 400 bp downstream of the amylase terminal codon.

[0091] The two oligonucleotides have sequences recognised respectively by the restriction enzymes *EcoRI* and *XbaI*. Both primers (40 pmoles) were used for a PCR reaction with the gDNA from *Aspergillus oryzae* MUCL 14492. The PCR reaction contained 2.5 U *Pfu* DNA polymerase (Stratagene), 20 mM Tris-HCl pH 8.0, 10 mM KCl, 2 mM MgCl<sub>2</sub>, 6 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% Triton X-100, 1 µg BSA into a final volume of 100 µl. After denaturation of the DNA during 4 min at 94°C, the reaction was performed with 20 cycles of 30 s at 94°C, 30 s at 55°C and 60 s at 72°C followed by an elongation step of 7 min at 72°C. The amplified DNA fragments were purified in a final volume of 50 µl with the QIAquick PCR purification kit (Qiagen) according to the manufacturer's protocol. The purified fragment was treated with the *EcoRI* and *XbaI* restriction enzymes. The reaction was performed with 5 U *Xba I* and 5 U *EcoR I* (Pharmacia), 1 x One-Phor-All buffer PLUS in 60 µl at 37°C overnight. The restricted fragments were purified with the QIAquick gel extraction kit (Qiagen) after electrophoresis on an agarose gel and eluted into 30 µl water.

[0092] The PCR fragment was inserted between the *EcoR I* and *Xba I* sites of the pBluescript II SK(-) vector (Stratagene). The vector was prepared as follows: 0.5 µg pBluescript II SK was treated by 5 U *EcoR I* and 5 U *Xba I* restriction enzymes (Pharmacia) in a 20 µl final volume, 2 x One-Phor-All buffer PLUS at 37°C overnight. After electrophoresis in an agarose gel, it was purified with QIAquick gel extraction kit (Qiagen) and eluted in 30 µl water. 2 µl of the PCR fragment was ligated to this vector (1 µl) in the presence of 1 mM ATP, 1U T4 DNA ligase (Pharmacia) and 1 x One-Phor-All buffer PLUS in a 10 µl final volume at 16°C overnight. The ligation product (1µl) was

electroporated into competent DH10b cells (BRL-Gibco) after dialysis against water. The new plasmid was termed pAMY3.

[0093] The promoter of the glyceraldehyde-3-P dehydrogenase gene from *Aspergillus nidulans* was inserted in front of the amylase gene. This promoter allows a rather strong constitutive transcription of genes located downstream of it (Punt, P.J., Dingemanse, M.A., Kuyvenhoven, A., Soede, R.D.M., Pouwels, P.H. and van den Hondel; C.A.M.J.J., 1990. Functional elements in the promoter region of the *Aspergillus nidulans* *gpdA* gene coding for the glyceraldehyde-3-P dehydrogenase. Gene. 93: 101-109 ; Punt, P.J., Zegers, N.D., Busscher, M., Pouwels, P.H. and van den Hondel; C.A.M.J.J., 1991. Intracellular and extracellular production of proteins in *Aspergillus* under the control of expression signals of the highly expressed *Aspergillus nidulans* *gpdA* gene. J. Biotechnol. 17: 19-34). To insert the amylase gene downstream of the GPD promoter, the plasmid pFGPDGLAT2 was digested with the restriction enzyme *NcoI*. After purification with the QIAquick gel extraction kit (Qiagen), the plasmid was blunted with T4 DNA polymerase and dNTP, purified, and restricted with *XbaI* restriction enzyme (5U) in the presence of 1 x One-Phor-All buffer PLUS in a 20 µl final volume 37°C overnight). The same protocol was applied on pAMY3 except that the *NcoI* restriction enzyme was replaced by *EcoRI*. The fragments of interest were isolated after agarose gel electrophoresis, purified with QIAquick gel extraction kit (Qiagen) and collected separately in 30 µl water. The purified promoter and vector DNA fragment (1 µl) was ligated with the amylase gene and its terminator from pAMY3 (1 µl) in the presence of 1 mM ATP, 1U T4 DNA ligase (Pharmacia) and 1 x One-Phor-All buffer PLUS in a 10 µl final volume at 16°C overnight. The ligation product (1µl) was electroporated into competent

DH10b cells (BRL-Gibco) after dialysis against water. The new plasmid was named pGPD-AMY.

[0094] The GPD promoter, the amylase gene and its terminator have been isolated from pGPD-AMY and inserted again into a new pGPD-AMY in order to have two copies of the functional gene on one plasmid. The first step was to digest pGPD-AMY with 1U *HindIII* restriction enzyme and 1 x One-Phor-All buffer PLUS in a 40  $\mu$ l final volume at 37°C overnight. After purification with QIAquick gel extraction kit (Qiagen), the linear DNA fragment was blunted in the presence of T4 DNA polymerase and dNTP. The plasmid was again purified with QIAquick gel extraction kit (Qiagen) and treated with *NotI* restriction enzyme and 1 x One-Phor-All buffer PLUS in a 40  $\mu$ l final volume at 37°C overnight. The fragment containing the functional amylase gene was finally isolated on agarose gel after electrophoresis. The pGPD-AMY plasmid (1 $\mu$ g) was digested by *XbaI* (1U) and 1 x One-Phor-All buffer PLUS in a 40  $\mu$ l final volume at 37°C overnight. After purification with QIAquick gel extraction kit (Qiagen), the linear DNA fragment was blunted in the presence of T4 DNA polymerase and dNTP. The plasmid was again purified with QIAquick gel extraction kit (Qiagen) and digested with the *NotI* restriction enzyme and 1 x One-Phor-All buffer PLUS in a 40  $\mu$ l final volume at 37°C overnight. The vector with the DNA fragment containing a functional amylase gene was isolated after agarose gel electrophoresis. Both fragments were ligated together in the presence of 1 mM ATP, 1U T4 DNA ligase (Pharmacia) and 1 x One-Phor-All buffer PLUS in a 10  $\mu$ l final volume at 16°C overnight. The ligation product (1 $\mu$ l) was electroporated into competent DH10b cells (BRL-Gibco) after dialysis against water. The new plasmid was termed p2GPD-AMY.

[0095] Finally a selection marker was inserted in p2GPD-AMY. The acetamidase gene will allow the growth of fungi on a

minimal medium with acetamide as unique nitrogen source. The functional acetamidase gene from *A. nidulans* (AmdS) has been isolated from 1  $\mu$ g of p3SR2 plasmid digested with the restriction enzymes *Sal*I (1U) and *Eco*RI (1U) and 2 x One-Phor-All buffer PLUS in a 40  $\mu$ l final volume at 37°C overnight. This DNA fragment was inserted between the *Sal*I and *Eco*RI restriction sites of pBluescript II SK. This new plasmid as well as p2GPD-AMY were digested with *Sal*I (or *Xba*I for p2GPD-AMY), blunted by treatment with the T4 DNA polymerase and digested with *Not*I. The functional AmdS gene DNA fragment and the openp2GPD-AMY plasmid were isolated from agarose after gel electrophoresis. Both fragments were then ligated together in the presence of 1 mM ATP, 1U T4 DNA ligase (Pharmacia) and 1 x One-Phor-All buffer PLUS in a 10  $\mu$ l final volume at 16°C overnight. The ligation product (1 $\mu$ l) was electroporated into competent DH10b cells (BRL-Gibco) after dialysis against water. The new plasmid was termed p2G-S.

#### 2.2.b.2. Cloning of the GPD promoter

##### Design of the oligonucleotides

[0096] Based on published sequences of fungal glyceraldehyde-3-P dehydrogenases (gpd), a set of four oligonucleotides has been designed in order to amplify a portion of the gpd gene of *Myrothecium*.

These oligonucleotides have the following sequences:

Gpd1 : 5' GGNATCAAYGGITTCGG 3'

Gpd2 : 5' GTGSWGSWGGGGATGATGTT 3'

Gpd3 : 5' GGTCGTATCGTNTTYCGIAAYGC 3'

Gpd4 : 5' GGAGCCAGGCAGTTGGTIGTRCA 3'

Where N stands for A,T, C or G; Y stands for C or T; I stands for dInosine; S stands for G or C; W stands for A or T; R stands for A or G.

#### Preparation of the genomic DNA

[0097] The genomic DNA of the following strains has been prepared using the Dneasy Plant Mini Kit (Qiagen): MUCL39210, MUCL11831, CBS449.71, IMI140595 and IMI290405.

#### PCR

[0098] PCR reactions have been performed in 0.25 ml tubes with a MJ research PTC-200 Peltier Thermal Cycler. The reaction mix (25  $\mu$ l) contained about 20 ng genomic DNA, 10 pM oligonucleotides, dNTP 200  $\mu$ M, 1 x buffer (Tris-HCl 10 mM, pH 8.3, MgCl<sub>2</sub> 1.5 mM, KCl 50 mM, BSA 0.1 ng/ml) and 1 unit of Taq polymerase (Invitrogen). Amplification conditions were the following: initial denaturation (94°C, 4 min); 25 cycles of denaturation (94°C, 30 sec)-hybridisation (52°C, 30 sec)-elongation (72°C, 1 min); final elongation (72°C, 7 min).

[0099] Cloning of the *gpd* gene of the strains MUCL11831, CB449.71 and IMI290405.

[0100] DNA fragments amplified using the protocol described above (with the oligonucleotides *gpd1* and *gpd2* as amplification primers) have been separated by agarose gel electrophoresis and purified with the aid of a QIAquick Gel Extraction Kit (Qiagen). A second round of PCR has been performed on these purified fragments and the resulting DNA fragments have been directly ligated into the plasmid commercially available pT-Adv (Advantage PCR cloning Kit - Clontech) using the recommendations of the manufacturer.

Ligations products have been used to transform competent cells of *Escherichia coli* DH5 $\alpha$  (Invitrogen). Positive clones have been identified by plasmid isolation and analysis by restriction enzyme digestion and agarose gel electrophoresis.

[0101] Sequencing of the *gpd* genes of the strains MUCL11831, CB449.71 and IMI290405.

[0102] The insert of the plasmid described above has been sequenced using usual procedures.

[0103] The sequence of the *gpd* gene of the strain MUCL11831 is shown in SEQ ID NO11.

[0104] The sequence of the *gpd* gene of the strain CBS449.71 is shown in SEQ ID NO12.

[0105] The sequence of the *gpd* gene of the strain IMI290405 is shown in SEQ ID NO13.

#### Cloning of the *gpd* gene of MUCL39210

[0106] The same procedure as for the cloning of the *gpd* genes of the strains MUCL11831, CB449.71 and IMI290405, except that the first PCR was performed using the oligonucleotides *gpd*2 and *gpd*3 and that the second PCR was performed with the oligonucleotides *gpd*3 and *gpd*4.

#### Sequencing of the *gpd* gene of the strain MUCL39210.

[0107] The inserts of 8 different inserts of the plasmids obtained above have been sequenced using usual procedure.

[0108] A consensus sequence of the *gpd* gene of the strain MUCL39210 is shown in SEQ ID NO14.



## Results

[0109] The MUCL39210 GPD partial sequence shown in SEQ ID NO14 was translated into a putative protein sequence as presented in Figure 2. The GPD partial sequence comprises a large putative intron. The intron sequence does not show homology to any protein from protein databases when translated from the six possible DNA frames. The putative protein sequence (SEQ ID NO15) shows the highest similarity with the GPD protein sequence of *Neurospora crassa* using the BLASTP 2.2.4. program against the non-redundant GenBank database as of 20th Augustus 2002. Between the various *Myrothecium* GPD protein sequences, the identity is 88% as shown in figure 3.

### 2.3. Transformation

#### 2.3.a. Transformation of *Myrothecium*

[0110] The strains *Myrothecium gramineum* (syn. *Xepiculopsis graminea*) MUCL39210, *Myrothecium gramineum* CBS449.71, IMI140595, IMI290405, and *Myrothecium verrucaria* CBS328.52 were transformed by generating protoplasts according to the protocol described by Punt et al. (Punt et al. 1992. Transformation of filamentous fungi based on hygromycin B and phleomycin resistance markers. Meth. Enzymol. 216:447).

[0111] The strains were grown in 350 ml Rich Liquid medium (malt extract 2%, peptone 1% and glucose 3%) at 25°C for a period between 16 and 36 hours depending on the strain. The culture was filtered through a Miracloth filter to collect the mycelium. The mycelium was washed with the OSM solution (CaCl<sub>2</sub> 0.27 M, NaCl 0.6 M) and then incubated with 20 ml OSM solution/g mycelium supplemented with 12 mg/ml β-D-glucanase (Interspex),

7.5 mg/ml driselase (Interspex) and 81.4 U/ml yeast lytic enzyme (ICN). The incubation is done at 25°C with slow agitation (80 rpm). [0112] The apparition of the protoplasts is followed visually under the microscope and varies from 90 to 180 min, depending on the strain. Once the protoplasts were formed, the suspension was putted on ice. The protoplasts were separated from intact mycelium by filtration through a sterile Miracloth filter and diluted with 1 volume STC1700 solution (sorbitol 1.2 M, Tris-HCl pH 7.5 10 mM, CaCl<sub>2</sub> 50 mM, NaCl 35 mM) or GTC (glucose 1 M, CaCl<sub>2</sub> 50 mM, Tris 10 mM pH8). The protoplasts were then collected by centrifugation at 2000 rpm, 10 min, 4°C and washed twice with STC1700 or GTC solution. They were finally resuspended in 100 µl of STC1700 or GTC (10<sup>8</sup> protoplasts/ml).

[0113] To obtain transformants resistant to hygromycine, c.a. 2 µg pCSN43 plasmid DNA and c.a. 8 µg p2G-S plasmid DNA were added to the protoplasts suspension.

To obtain transformants able to grow on acetamide as sole nitrogen source 3 µg p3SR2 plasmid DNA and 9 µg p2G-S plasmid DNA were added to the protoplasts suspension.

[0114] After 30 min at 20°C, 250, 250 and 850 µl PEG solution (PEG 4000 60%, Tris-HCl 10 mM pH 7.5 and CaCl<sub>2</sub> 50 mM) were added successively and the suspension was further incubated for 20 min at 20°C. PEG treated protoplasts suspension was diluted by the addition of 50 ml STC1700 or GTC and centrifuged 5 min at 4°C, 2000 rpm. The protoplasts were then resuspended in 200 µl STC 1700 or GTC and plated onto selective medium.

[0115] For hygromycin selection Malt Extract Agar Medium or Potato Dextrose Agar osmotically stabilised with 1.2 M sorbitol or 1 M glucose and supplemented with 500 µg/ml hygromycin was used. In some cases, protoplasts are plated onto the same medium without hygromycin. After 2 hours incubation,

hygromycin (500  $\mu$ g/l) overlays (8 ml molten agar) were dispensed over the protoplasts.

[0116] For acetamidase selection the AMM medium (Pontecorvo, G., Roper, J.A., Hemmons, L.M., Mc Donald, K.D. and Bufton, A.W.J., 1953. Adv. Genet. 5: 142-152.) supplemented with sorbitol 1.2M and acetamide as sole nitrogen source was used.

[0117] A series of transformants has been obtained for each strain and for each transformation trial. These transformants have been replicated on the same selection medium without sorbitol and kept for further analysis.

#### 2.3.b. Control transformation.

[0118] The same protocol as described in 2.3.a. has been used to transform the MUCL28817 strain of *Aspergillus niger*. A number of transformants have been selected and kept for further analysis.

### EXAMPLE 3.

#### Analysis of the transformants

##### 3.1. Amplification of integrated DNA

##### 3.1.a. Preparation of genomic DNA from fungal strains

[0119] The genomic DNA of the transformed fungal strains as well as the control strains has been prepared using the Dneasy Plant Mini Kit (Qiagen).

### 3.1.b. PCR reactions

[0120] According to the published sequence of the *E. coli* hygromycin resistance gene (hph gene), the oligonucleotides hphpCSN431 (5'-ATGCCTGAACTCACC GCGACG-3') and hphpCSN432 (5'-CTATTCCTTTGCCCTCGG-3') were designed to amplify a region of 1020 bp comprising the hph gene.

[0121] To detect the presence of p2G-S into the *Myrothecium* genome without amplification of the endogenous amylase gene or the GPD promoter, the GPD2 (5'-TCTGGCATGCGGAGAG-3') and AMY5 (5'-CGATGATGCCCTGCCA-3') oligonucleotides were designed to allow the amplification of a 1134 bp length fragment covering a part of the *A. niger* GPD promoter fused to a part of the sequence of the TAKA amylase gene.

[0122] PCR reactions have been performed in 0.25 ml tubes with a MJ research PTC-200 Peltier Thermal Cycler. The reaction mix (25  $\mu$ l) contained about 20 ng genomic DNA, 10 pM oligonucleotides, dNTP 200  $\mu$ M, 1 x buffer (Tris-HCl 10 mM, pH 8.3, MgCl<sub>2</sub> 1.5 mM, KCl 50 mM, BSA 0.1 ng/ml) and 1 unit of Taq polymerase (Invitrogen). Amplification conditions were the following: initial denaturation (94°C, 4 min); 25 cycles of denaturation (94°C, 30 sec)-hybridisation (55°C, 30 sec)-elongation (72°C, 1 min); final elongation (72°C, 7 min).

### 3.1.c. Results

[0123] The results of the analysis of 5 different transformants of *Myrothecium gramineum* (syn. *Xepiculopsis graminea*) MUCL39210 selected for the hygromycin resistance and amylase production are shown on figure 4. The agarose gel electrophoresis of the PCR products shows that all the five transformants studied have integrated both the selection marker

gene (hph gene - c.a. 1000 bp DNA fragment - lanes 4, 6, 8, 10 & 12) and the reporter gene ( $\alpha$ -amylase gene of *Aspergillus oryzae* - c.a. 1100 bp DNA fragment - lanes 5, 7, 9, 11 & 13). Negative controls are presented in the lanes 2 & 3 (respectively products of hygromycine and  $\alpha$ -amylase genes PCR amplification). Lanes 14 and 15 are positive controls (PCR reactions performed respectively on the pCSN43 and p2G-S plasmids).

### 3.2. Evaluation of recombinant protein production

#### 3.2.a. Culture of the transformants

[0124] Twelve transformants of *Myrothecium gramineum* (syn. *Xepiculopsis graminea*) MUCL39210 obtained by transformation with the plasmids pCSN43 and p2G-S as well as 49 transformants of *Aspergillus niger* MUCL28817 have been cultivated in AMM medium with 3% sucrose as carbon source in shaking incubators (140 rpm) at 25°C. The culture volume was 20 ml for a total erlenmeyer volume of 100 ml.

After 4 days, the supernatants of the cultures were collected by centrifugation.

#### 3.2.b. $\alpha$ -amylase activity determination

[0125] The  $\alpha$ -amylase activity was determined by a modification of the method included in the Sigma Amylase kit. The substrate was diluted in a 100 mM  $\text{NaH}_2\text{PO}_4$  buffer pH 6.0. One amylase unit was defined as the amount of enzyme that gives an absorbance of 1 OD unit at 405 nm after 15 min reaction at 37°C and when 120  $\mu\text{l}$  of the reaction mix are read in a 96 holes microplate with the model 550 microplate reader (Bio-Rad).





Tf. 45			2,275	147,5
Tf. 46			1,525	105
Tf. 47			1,475	90
Tf. 48			27,75	1420
Tf. 49			0,75	47,5
Average	28,8	2263	10,48	562,5

[0127] The table 1 shows that all transformants obtained with the strain *Myrothecium gramineum* (syn. *Xepiculopsis graminea*) MUCL39210 produce significantly more  $\alpha$ -amylase than a non-transformed control strain. Many of the *Aspergillus niger* MUCL 28817 transformants produce very few  $\alpha$ -amylase compared to the non-transformed strain indicating that in those cases, they should not have been transformed by the reported gene or that this reporter gene is integrated under a non-expressible form.

[0128] The *Myrothecium* transformants produce more  $\alpha$ -amylase than the *Aspergillus* transformants obtained and cultivated in the same conditions. The best *Xepiculopsis* transformant (n°9) produces more than twice  $\alpha$ -amylase compared to the best *Aspergillus* transformant (n°29). On an average the *Myrothecium* transformants produce 2.75 more  $\alpha$ -amylase than the *Aspergillus* transformants.

[0129] Furthermore, the *Myrothecium* transformants have a better specific production of  $\alpha$ -amylase (amount of enzyme / g of biomass) compared to the *Aspergillus* transformants.

#### 3.4. Stability of the transformants

[0130] A hygromycin-resistant transformant of *Myrothecium gramineum* (syn. *Xepiculopsis graminea*) MUCL39210 was stored at -



80°C. During the storage, the same transformant was successively replicated 20 times on solid PDA (Potato Dextrose Agar) medium without selective pressure. After these replica platings, the two strains (the original transformant and the replicated strain) together with the non-transformed *Myrothecium gramineum* (syn. *Xepiculopsis graminea*) MUCL39210 were grown (24°C - 145 rpm) in 20 ml of liquid medium (AMM medium supplemented with 3% sucrose and 0.5% yeast extract, pH 6.3) in 100 ml erlenmeyers. Samples of the cultures were taken at various times and assayed for  $\alpha$ -amylase activity.

Table 2

	Activity U/ml	
	120 HF	144 HF
Wild-type	3,225	9,4
Original transformant	15,9	19,575
Transformant replicated 20 times	16,925	21,375

As shown in the table 2, the number of replications performed with the *Myrothecium gramineum* (syn. *Xepiculopsis graminea*) MUCL39210 transformant does not affect the productivity of the strain.

### 3.5. Analysis of transformants of other *Myrothecium* strains

[0131] Similar results (enzymatic activity determination, control of the integration of the DNA constructs) as the ones obtained for transformants of *Myrothecium gramineum* (*Xepiculopsis graminea*) MUCL39210 have been obtained with transformants of another *Myrothecium* strain, i.e. CBS449.71.

[0132] The strain CBS 449.71 and its transformants were grown (30°C - 145 rpm) in 70 ml of liquid medium (AMM medium supplemented with 3% sucrose and 0.5% yeast extract, pH 6.3) in 250 ml Erlenmeyer. The amylase activity was measured after 123 hours fermentation (Table 3).

Table 3

strain	Amylase activity after 123HF
CBS 449.71 wt.	10
CBS 449.71 transformant n°5	11,675
CBS 449.71 transformant n°7	17,7

SEQUENCE LISTING

SEQ ID NO1

28s rDNA sequence of the strain MUCL39210

CAGGGATTGCCTCAGTAACGGCGAGTGAAGCGGCAACAGCTCAAATTTGAAATCTGGCCCCCG  
 GCCCGAGTTGTAATTTGCAGAGGATGCTTTTGGCGAGGTGCCTTCCGAGTTCCCTGGAACGGGA  
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 TGGGCCATTTTGGTAAGCAGAACTGGC

SEQ ID NO2

28s rDNA sequence of the strain MUCL11831

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 AGCACGTGAAATTGTTAAAAGGGAAGCGTTTACGACCAGACTTGTGCCGGTTGATCATCCAGCG  
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 GGGCCATTTTTGGTAA

SEQ ID NO3

28s rDNA sequence of the strain CBS449.71

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 TCGTCTTCGTATGCGAGTGTTTCGGGTGTAAACCCCTACGCGTAATGAAAGTGAACGCAGGTGA  
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SEQ ID NO4

28s rDNA sequence of the strain IMI140595

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SEQ ID NO5

28s rDNA sequence of the strain IMI290405

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SEQ ID NO6

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SEQ ID NO7

ITS sequence of the strain MUCL11831

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CCTCGGATCAG

SEQ ID NO8

ITS sequence of the strain CBS449.71

GCGGGACCGCCCCGGCGCCTTCGGGCCCGGAACAGGCGCCCGCGGAGGCCCAAACCTTTAT  
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SEQ ID NO9

ITS sequence of the strain IMI140595

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SEQ ID NO10

ITS sequence of the strain IMI290405

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SEQID NO11

DNA sequence of the gpd gene of the strain MUCL11831

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DNA sequence of the *gpd* gene of the strain CBS449.71

GTGCAGCTGGGGATGAIGTTCTTGGGCAGCACCACGGCCACCCGCGCCAGTCCTTTGGCGGAGGGAC  
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CGTGCTCGACGGCGTTGCGGAAGACGATACGACCAATGCGGCGCGAACCCATTGATCCC

DNA sequence of the *gpd* gene of the strain IMI290405

GTGGTGGAGGGGATGATGTTCTGGGCAGCACACCAGGCCACCACGCCAGTCTTGGCGGAGGGAC  
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ATGAAGGGATCGTTGACGGCAACGATCTCGACGTCGGAGTGCTCGACGGCGTTGCGGAAGACGA  
TACGACCAATGCGGCCGAACCCATTGATACC

SEQ ID NO14

Sequence of the *gpd* gene of *Myrothecium gramineum* (*Xepiculopsis graminea*) MUCL39210

GCCGTCGAGCACTCCGACGTCGAGATCGTTGCCGTCAACGACCCCTTCATTGAGCCCAAGTACG  
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CCGTCGATGGCGATGACCTGACCGTCAACGGCAAGAAGGTCCGCTTCTACACTGAGCGTGACCC  
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CATCTCCAACGCTTCTTG



ABSTRACT**MYROTHECIUM SP. TRANSFORMATION AND EXPRESSION SYSTEM**

The present invention is related to a transformation and an expression system in which Myrothecium sp. host cells are used to express homologous or heterologous proteins or are used to genetically engineer metabolic pathways.

S:\DOCS\DOH\DOH-6969.DOC  
083002

## Bootstrap ITS

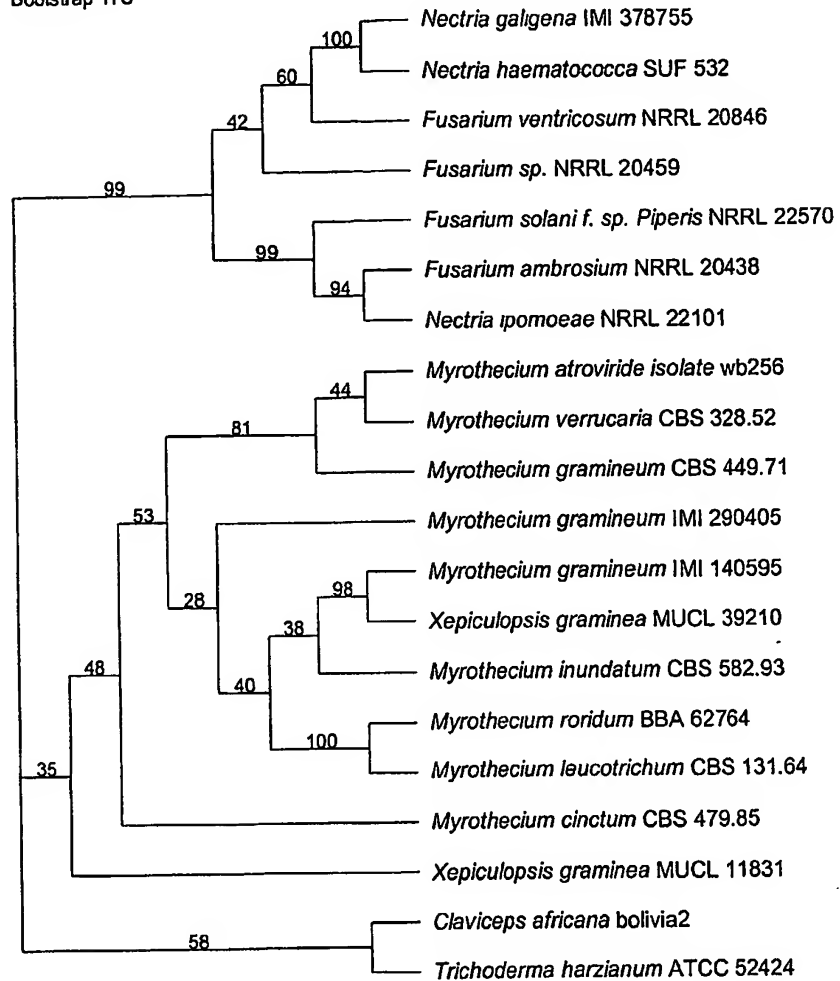


FIGURE 2

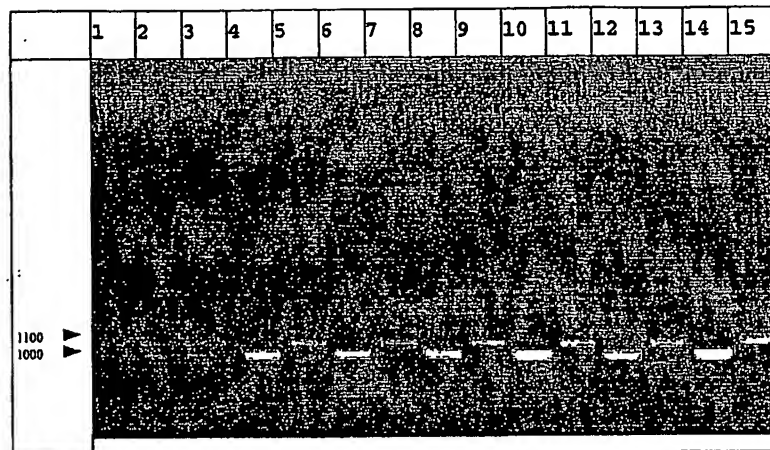
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 -----  
 T K D K A A A H L K G G A K K V I I  
 765 774 783 792 801 810  
 TCT CTG CCC CCT CTG CCG ATG CCC CCA TGT ACG TTA TGG GTG TCA ACG AGG AGA  
 -----  
 S A P S A D A P M Y V M G V N E E T  
 819 828 837 846  
 CCT ACG ACG GCA GCG CCG ACG TCA TCT CCA ACG CTT CTT G 3'  
 -----  
 Y D G S A D V I S N A S

```

MUC11831      GINGFGRIGRIVFRNAVEHDPDIEIVAVNDPFIETKYA/AYMLKYDSTHGLFKGFEVADGAD
CBS449 71     GINGFGRIGRIVFRNAVEHDDVEIVAVNDPFIETKYA/AYMLKYDSTHGLFKGFEVSDGAD
IMI290405     GINGFGRIGRIVFRNAVEHSDVEIVAVNDPFIETKYA/AYMLKYDSTHGVFKGEVTVDGAD
MUC139210     -----AVEHSDVEIVAVNDPFIETKYA/AYMLKYDSTHGLFKGEVTVDGD
                *****:*****..* *****:***** **
MUC11831      LSVNGKKVRFYTERDPASIPWKETGAEYIVESTGVFTTTDKAAHLAGGAKKVIISAPSA
CBS449 71     LTVNGKKVRFYTERDPAIPWKETGAEYIVESTGVFTTKDKAAHLKGGAKKVIISAPSA
IMI290405     LTVNGKKVRFYTERDPAIPWKETGADYIVESTGVFTTKDKAAHLKGGAKKVIISAPSA
MUC139210     LTVNGKKVRFYTERDPAIPWKETGAEYIVESTGVFTTKDKAAHLKGGAKKVIISAPSA
                *:*****:*****:*****.*** ** *****
MUC11831      DAPMYVMGVNEKTYDGSADVISNASCTTNCLAPLAKVLNDKYTIIIEGLMTTVHSYTATQK
CBS449 71     DAPMYVMGVNEETYDGSADVISNASCTTNCLAPLAKVIHDKFTIIIEGLMTTVHSTLPPR
IMI290405     DAPMYVMGVNEETYDGSADVISNASCTTNCLAPLAKVIHDKFTIIIEGLMTTVHSYTATQK
MUC139210     DAPMYVMGVNEETYDGSADVISNAS
                *****
MUC11831      TVDGPSAKDWRGGRGAAQNIIPPT
CBS449 71     PLTVPPPTGAVAVVLPTSSPAA
IMI290405     TVDGPSAKDWRGGRGAAQNIIPST
MUC139210     -----

```

FIGURE 4



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